Molecular analysis of the 21-kb bacteriocin-encoding plasmid pEF1 from *Enterococcus faecium* 6T1a

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ABSTRACT

The complete 21,344-bp DNA sequence of the non-conjugative, bacteriocin-encoding plasmid pEF1 from *Enterococcus faecium* 6T1a was determined. Forty-six putative open reading frames (ORFs) which could code for proteins longer than 42 amino acids were found. Those included the structural genes for the previously described bacteriocins enterocin I and J (also named as enterocins L50A and L50B). After comparison to sequences in public databases, analysis of the gene organization of pEF1 suggests a modular structure with three different functional domains: the replication region, the bacteriocin region and the mobilization plus UV-resistance region. This genetic mosaic structure most probably evolved through recombination events promoted by transposable elements. The hypothesis that the bacteriocin cluster on pEF1 could act as a functional plasmid stabilization module in *E. faecium* 6T1a is discussed.

Keywords: bacteriocinogenic plasmid; Plasmid stability; *Enterococcus faecium*; Nucleotide sequence; Annotation.

INTRODUCTION

Lactic acid bacteria (LAB) associated with foods and beverages have been generally recognized as safe (GRAS) microorganisms for a long period of time (Klaenhammer et al., 2005). Enterococcal species of LAB have been considered as harmless commensal gut microorganisms, although more recently strains of *Enteroccus faecium* and *Enterococcus faecalis* have been acknowledged as leading causes of hospital-acquired infections (Emori and Gaynes, 1993; Schwarz et al., 2001;Grady and Hayes, 2003). This is especially relevant as enterococci are intrinsically resistant to many antibiotics and can acquire further resistance through conjugative plasmids and transposons which can also donate to other bacterial species (Schwarz et al., 2001).

Plasmids, as extrachromosomal, self-replicative and often transmisible genetic elements, encode many characteristics that may represent selective advantages for the cells harbouring them. Among these, antibiotic and bacteriophage resistance, sugar and amino acid metabolism, production of proteolytic enzymes, and bacteriocin production and immunity have been described (Dougherty et al., 1998; Martínez-Bueno et al., 1998; Cintas et al., 1998; Floriano et al, 1998; van Kranenburg et al., 2005). However, most plasmids remain cryptic, as molecular and phenotypic characterization has not been carried out routinely untill very recently. This is especially true for large plasmids, whose study is being benefit from standard genomic techniques developed for the chromosome studies (van Kranenburg et al., 2005).

In a previous work, it was shown that the ability to produce and be immune to a bacteriocin named enterocin I by the strain *E. faecium* 6T1a was linked to a 23-kb plasmid harboured by such strain (Floriano et al., 1998). This plasmid was designated pEF1, and

only a 2.5-kb DNA sequence was known at that time (EMBL acc. no. **Y16413**). In this report, we describe the complete molecular analysis of plasmid pEF1, which resembles a combination of different plasmid and chromosomal DNA fragments of mostly *E. faecium* and *E. faecalis* origin linked through insertional or conjugative events in a self-replicative unit.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. faecium 6T1a, an enterocin I and J producer, was isolated from an olive fermentation in southern Spain and described previously (Floriano et al., 1998). *E. faecalis* EFS-2, an enterocin AS-48 producer, was kindly provided by Dr. Martínez-Bueno from the University of Granada, Spain. They were propagated in MRS broth (Oxoid, Basingstoke, England) at 37 ºC. *Escherichia coli* DH5α was cultivated in LB broth (Sambrook et al., 1989), aerobically at 37 ºC.

Bacteriocin assays

The bacterial strain to be tested for bacteriocin production was streaked onto MRS-agar plates to obtain isolated colonies. The plates were incubated at 37 ºC for 16 h and then overlaid with 4.5 ml MRS soft–agar inoculated with ca. 10^5 cfu/ml of the indicator strain. These plates were further incubated at 37 ºC for 24 h, untill lawns of the indicator strain were grown, allowing the detection of inhibition halos surrounding the isolated colonies. Alternatively, 10-µl drops of semi-purified AS-48 (kindly provided by Dr. Martínez-

Bueno) were spotted onto lawns of indicator strains prepared as described above. These plates were incubated at 37 ºC for 24 h and resulting inhibition halos recorded. *E. faecium* 6T1a and *E. faecalis* EFS-2 were both used as indicator strains.

DNA techniques

Total DNA was prepared from *E. faecium* as described previously (Cathcart, 1995), and plasmid purification was carried out by CsCl gradient centrifugation (Sambrook et al., 1989). Isolation of *E. coli* plasmid DNA and subsequent nucleic acid manipulations were carried out as described by Sambrook et al. (1989). Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics (Penzberg, Germany) and used according to the manufacturer instructions. Shrimp Alkaline Phosphatase (SAP) was purchased from USB Corporation (Cleveland, Ohio, USA) and used according to the manufacturer instructions.

PCR Amplifications

The Expand High Fidelity PCR System (Roche) was used to amplify the different DNA fragments from either pEF1 or the subsequent pUC18 subclones. Primers used in PCRs were synthesized by MWG Biotech (Ebersberg, Germany).

Sequencing of pEF1

Purified samples of pEF1 after CsCl centrifugation gradient were digested with *Hin*dIII, the resulting DNA fragments separated by size through 0.7 % agarose gels and then individually extracted and purified by the freeze-squeeze technique (Sambrook et al., 1989). Each DNA fragment was ligated to SAP-dephosphorilated, *Hin*dIII-digested pUC18

cloning vector and the mixtures were electroporated into *E. coli* DH5α. Both DNA strands of the inserts from every subclone obtained were initially sequenced using the universal, pUC18-derived primers. To complete the sequencing, the primer walking strategy with synthetic oligonucleotide primers was used. Posible gaps at the joints of consecutive subclones were filled-up by sequencing PCR-amplified DNA fragments overlapping such regions, using pEF1 DNA as the template in the corresponding PCRs.

Nucleotide sequencing was performed by the Servicio de Secuenciación Automática de ADN, CIB-CSIC (Madrid, Spain), with an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Perkin Elmer, Norwalk, Conn., USA).

Analysis of ORFs and amino acid alignments were performed using the programs from the Genetics Computer Group (University of Wisconsin, Madison, USA), the National Center for Biotechnology Information (NCBI, Bethesda, Madison, USA), and the EMBL-EBI tools (Cambridge, UK). Nucleotide sequences shown in this article have been deposited with the GenBank acc. no. **DQ198088**.

RESULTS AND DISCUSSION

General features of pEF1

Plasmid pEF1 consists of 21,344 bp and an average G+C content of 32 %. The C of the unique AvaI site was chosen as reference site (bp 1). Forty-six putative open reading frames (ORFs) which could code for proteins longer than 42 amino acids were found (Table 1), 27 of them on the clockwise (cw) and the other 19 on the counter-clockwise (ccw) strand (Figure 1).

Analysis of the gene organization of pEF1 suggests a modular structure, as it has been found for many other medium and large plasmids from Gram-positive bacteria that have been recently characterized: pMRC01 (60 kb, Dougherty et al., 1998); pRE25 (50 kb, Schwarz et al., 2001); pMD5057 (11 kb, Danielsen, 2002); pRUM (25 kb, Grady and Hayes, 2003); pIP501 (31 kb, Thompson and Collins, 2003); pCF10 (68 kb, Hirt et al., 2005); among others. Plasmid pEF1 appears to be divided into three different functional domains: the replication region, the bacteriocin region and the mobilization plus UV-resistance region.

The replication region

This region spans 3.7 kb, from co-ordinate 15,236 (the end of *prgO*) to 18,998 (the end of *prgN*), being virtually identical to a homologous region described in pRE25 (acc. no. **X92945**). From co-ordinate 18,400 to 19,000, overlapping the end of an ORF which is identical to *repR* from pRE25, a putative replication origin (oriR) is predicted. In this area, up to five putative DnaA boxes sharing high homology (maximum 2 mismatches) to the consensus 5'-TTATCCACA-3' (Meijer et al, 1995) are located, two of them in the cw and three in the ccw strand. Also, several inverted repeats having the potential to form stem-loops are found starting at positions 18,413 and 18,483 (10 bp, 1 mismatch), 18,844 and 18,538 (15 bp, 3 mismatches), 18,450 and 18,538 (18 bp, 4 mismatches), 18,655 and 18,673 (14 bp, 3 mismatches), and 18,811 and 18,971 (9 bp, 1 mismatch). In addition, significative direct repeats are found at positions 18,494 to 18,509 (8 bp, 2 repeats, 94 % identity [id.]), 18,682 to 18,709 (7 bp, 4 repeats, 75 % id.), 18,910 to 18,949 (8 bp, 5 repeats, 70 % id.), and 18,974 to 18,987 (7 bp, 2 repeats, 93 % id.). Finally, from co-

ordinate 18,570 to 18,612 there are 42 bp which are 75 % identical to the defined replication origin of pAMβ1 (acc. no. **AF007787**).

Considering all these data together, it is reasonable to predict that pEF1 is replicated using the product of *repR* and the oriR proposed above. Also, taking into account the significative homology of RepR to the replication proteins of plasmids such as pIP501 (acc. no. **X17655**) and pAMβ1 (50 % id., 67 % similarity [sim.]) and the size of the plasmid, it can be predicted that pEF1 replicates via the theta-type replication mechanism.

The bacteriocin region

Spanning 6.7 kb, from co-ordinate 2,212 (the end of *entJ*) to 8,936 (the end of *as48H*), this region contains the most interesting features of pEF1: bacteriocin production and immunity. Thus, the genes coding for enterocins I and J (identical to enterocins L50A and B from *E. faecium* L50 [Cintas et al., 1998], acc. no. AJ223633), two antilisterial bacteriocins that are secreted without a signal or leader peptide, are located in this fragment (Floriano et al, 1998). In the close vicinity but in the oposite direction, three clustered orfs are found which are virtually identical to *orfE*, *orfF* and *orfG* (*L50E*, *L50F* and *L50G*, respectively, in Figure 1) from pCIZ1, the enterococal plasmid coding for enterocins L50A and L50B. According to the authors (Drs. Pablo Hernández and Luis Cintas, Universidad Complutense, Madrid, personal communication), these ORFs could code for transporters of the ABC type. In fact, the product of *L50F* shares some homology (30 % id., 46 % sim.) to a putative transporter protein from *Staphylococcus saprophyticus* ATCC15305 (acc. no. BAE18108), while *L50G* shares also some homology (21 % id., 48 % sim.) to a membrane-spanning protein from *Bacillus cereus* ATCC14579 (acc. no. AAP08051).

Downstream of *L50G*, two striking putative orfs appear: *orf16* and *orf6*. None of the hypothetical proteins encoded by these ORFs shares any significant homology with proteins in the data banks. The G+C content of 25 and 19 % for *orf6* and *orf16*, respectively, is very low and quite appart from the average of the whole plasmid (32 %). Posible regulatory functions or the vestige of non-functional genes could be thinked of. Nevertheless, putative ribosomal binding sites are found inmediately upstream of *orf6* (co-ordinates 4,404 to 4,416) and *orf16* (co-ordinates 3,951 to 3,956). Also, tipical –10 and –35 regions are found upstream of *orf16* (co-ordinates 3,905 to 3,910 and 3,879 to 3,885, respectively). These two ORFs are also present in pCIZ1 at the same position, where their functionality remains unknown (Drs. Pablo Hernández and Luis Cintas, personal communication).

Finally, this bacteriocin region ends with four consecutive ORFs sharing high homology to the previously characterized operon *as-48EFGH*, involved in immunity to AS-48 (Díaz et al., 2003). To test wether this region confers immunity to AS-48 in *E. faecium* 6T1a, a bacteriocin assay with isolated colonies of *E. faecalis* EFS-2, an AS-48 producer, was carried out using *E. faecium* 6T1a as the indicator strain. Inhibition halos appeared around *E. faecalis* EFS-2 isolated colonies, thus demonstrating that the *as-48* operon in *E. faecium* 6T1a is not functional in the sense of confering immunity against AS-48. This conclusion was reinforced by the spot-on-lawn test using semi-purified AS-48, which rendered inhibition halos 16-mm in diameter on lawns of strain 6T1a. Controls using lawns of strain EFS-2 gave no inhibition at all.

The mobilization and UV-resistance region

This is an approximately 9-kb region whose initial structure seems to have been interrupted by the bacteriocin region described above through a putative insertional event carried out by an *IS981*-like element (Figure 1). It would span from the start of *orf9* (*efae2308*) at co-ordinate 19,099 to the end of *orf8* (*efae1887*) at co-ordinate 717, the first part; and from the start of *mobC* (co-ordinate 9,337) to the end of *orf17* (*efae2609*; co-ordinate 14,165), the second part. According to the homologies found in the data banks, most of the putative proteins encoded in this region show significative homology with hypothetical proteins annotated in the draft sequence of the *E. faecium* DO genome (Baylor College of Medicine; URL: http://www.hgsc.bcm.tmc.edu/projects/microbial/microbialblast.cgi?organism=Efaecium) (Table 1). At least four ORFs seem to have been directly involved in the mobilization of pEF1 in the past: *orf15 (efae1888), orf8 (efae1887), mobC* and *res* (Table 1). Thus, *orf15* shares 26 % id. and 53 % sim. to *ComGC* (acc. no. **AL935258**) from *L. plantarum* WCFS1, involved in competence; *orf8* shares 35 % id. and 54 % sim. to a putative relaxase/nickase encoded by plasmid pHTβ from *E. faecium* (acc. no. **AB183714**); *mobC* and *res* are virtually identical to a putative cell filamentation protein (acc. no. **CAC29207**) and a putative resolvase (acc. no. **CAC29208**), respectively, encoded by pRE25 from *E. faecalis* RE25. The products of *mobC* and *res* are also identical to the hypothetical proteins Efae657 (acc. no. **EAN08584**) and Efae658 (acc. no. **EAN08585**) from *E. faecium* DO.

Conjugation experiments carried out using *E. faecium* 6T1a as the donor strain did not resulted in DNA transfer when *E. faecium* 6T1a-20 (a pEF1-free strain derived from 6T1a), *E. faecalis* OG1X or *E. faecalis* JH2-2 were used as the recipient strains (data not

shown). This result, together with the lack of essential *tra* genes and an oriT sequence, suggests that pEF1 is not a conjugative plasmid in its present configuration.

In addition, this region includes a putative UV-resistance gene highly homologous to *uvrA* described in pRUM from *E. faecium* (acc. no. **AAO52829**), which shares 51% id. and 71 % sim. to a structural gene for UV resistance (acc. no. **BAA23799**) from pAD1, harboured by a strain of *E. faecalis* (Francia et al., 2001).

CONCLUSIONS

The complete sequence of the bacteriocin-encoding plasmid pEF1 reveals a genetic mosaic structure which most probably evolved through recombination events promoted by transposable elements. Being at present a medium-sized plasmid, it appears as if conjugational properties were lost when a bacteriocin-production/immunity cassette was inserted into the ancient, larger structure of pEF1. It has been postulated and, in a few cases demonstrated, that bacteriocin production confers a competitive advantage to the host strain (Ruiz-Barba et al., 1994; Smith and Dworkin, 1994; Dykes, 1995). This fact could overcome the burden of plasmid carriage, resulting in an enhanced fitness of the host relative to the same host after plasmid curing (Lenski et al., 1994; Dykes and Hastings, 1997). Actually, plasmid-free derivatives of the strain 6T1a, no longer producing bacteriocin, become also sensitive to enterocin I/J (Floriano et al., 1998). In this sense, the bacteriocin cluster on pEF1 could act as a functional plasmid stabilization module in *E. faecium* 6T1a, as it has been demonstrated for the Axe-Txe, toxin-antitoxin system in pRUM, a plasmid from a clinical isolate of *E. faecium* (Grady and Hayes, 2003). On the other hand, it has been proposed that the likelihood of the coevolution of non-conjugative

bacteriocin-encoded plasmids and their hosts to beneficial associations is high (Dykes and Hastings, 1997).

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LEGENDS OF THE FIGURES

Figure 1. Circular map of plasmid pEF1. Putative ORFs are shown either by their respective number or the name of the most significant homologue in the public data bases (see Table 1 for further details). Predicted functional regions and color code: replication; bacteriocin production and immunity; mobilization and UV resistance.

Figure 1

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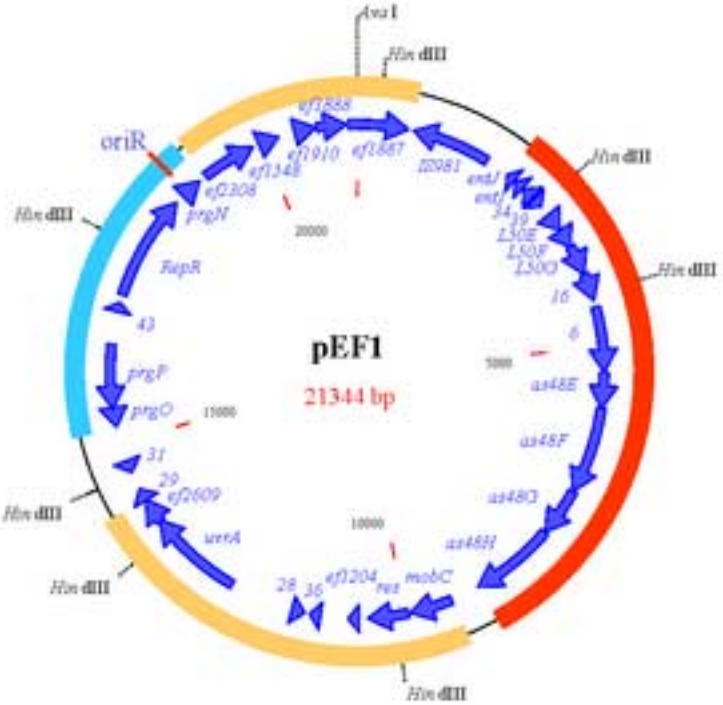

Table 1

Table 1. Open reading frames and amino acid similarity of putative proteins encoded by plasmid pEF1 to sequences in public databases

orf	gene	5'	3'		aas.	%G+C	Closest homologue (organism)	% id/sim	Acc. No.	pEF1 range	Homologue range (total aas)
1	*repR*	17074	18567		497	37	RepR, putative Rep protein of pRE25 (*E. faecalis*).	99/99	CAC29157	1-497	1-497 (497)
2	*uvrA*	12473	13822		449	38	UvrA, putative UV-resistance gene of pRUM (*E. faecium*)	95/98	AAO52829	9-449	1-441 (441)
3	*as-48F*	5843	7060		405	32	as-48F, enterocin AS-48 cluster (*E. faecalis*)	75/87	CAD27715	3-405	4-407 (407)
4	*as-48H*	7737	8936		399	33	as-48H, enterocin AS-48 cluster (*E. faecalis*)	88/94	CAD27717	1-399	1-399 (399)
5	*IS981*	779	1879	C[a]	366	34	Transposase of the IS981 series (*L. lactis)*	49/68	AAK06124	100-363	11-271 (279)
6		4437	5351		304	25	No hits				
7	*prgP*	15483	16382	C	299	35	PrgP, involved inreplication of pRE25 (*E. faecalis*)	100/100	CAC29215	1-299	19-317 (317)
8	*efae1887*	21222	717		279	36	Hypothetical protein EfaeDRAFT 1887 (*E. faecium*)	95/97	EAN10139	1-277	1-277 (530)
9	*efae2308*	19099	19869		256	34	Hypothetical protein EfaeDRAFT 2308 (*E. faecium*)	97/97	EAN10371	1-241	1-241 (283)
10	*as-48G*	7057	7740		227	33	as-48G, enterocin AS-48 cluster (*E. faecalis*)	92/96	CAD27716	1-227	1-227 (227)
11	*mobC*	9337	9942		201	34	Putative cell filamentation protein of pRE25 (*E. faecalis*)	99/100	CAC29207	1-201	1-201 (201)
12	*res*	9958	10530		190	40	Putative resolvase of pRE25, Tn3 family (E. faecalis)	100/100	CAC29208	1-190	1-190 (190)
13	*as-48E*	5353	5850		165	26	as-48E of the enterocin AS-48 cluster (*E. faecalis*)	44/70	CAD27714	1-164	1-164 (169)
14	*L50G*	3537	3968		143	24	orfG, enterocin L50 cluster (*E. faecium*)	98/99	CAA11517	1-108	1-108 (108)
15	*efae1888*	20780	21202		140	32	Hypothetical protein EfaeDRAFT 1888 (*E. faecium*)	77/85	EAN10140	1-140	1-140 (140)
16		3965	4351		128	19	No hits				
17	*efae2609*	13788	14165		125	38	Hypothetical protein EfaeDRAFT 2609 (*E. faecium*)	88/93	AAO52828	1-125	1-125 (125)
18	*prgN*	18681	18998		105	38	PrgN, involved in conjugationl of pRE25 (*E. faecalis*)	100/100	CAC29158	1-98	1-98 (98)
19	*efae1910*	20470	20778		102	29	Hypothetical protein EfaeDRAFT 1910 (*E. faecium*)	57/79	EAN10162	1-94	1-90 (92)
20	*efae1548*	19951	20250		99	33	Hypothetical protein EfaeDRAFT 1548 (*E. faecium*)	27/55	EAN10004	57-99	219-261(665)
21	*prgO*	15236	15511	C	91	34	PrgO, involved in replication of pRE25 (*E. faecalis*)	100/100	CAC29214	1-91	1-91 (91)
22	*L50F*	3275	3547		90	28	orfF, enterocin L50 cluster (*E. faecium*)	100/100	CAA11518	1-90	1-90 (90)
23	*L50E*	3005	3274		89	25	orfE, enterocin L50 cluster (*E. faecium*)	100/100	CAA11519	1-89	1-89 (89)
24		10781	11038	C	85	29	No hits				
25		419	661	C	80	34	No hits				
26		12556	12762	C	68	41	No hits				
27		6907	7113	C	68	32	No hits				
28		11397	11585	C	62	31	No hits				
29		14147	14332		61	38	No hits				
30		8162	8344	C	60	34	No hits				
31		14576	14755	C	59	36	No hits				
32		6616	6786	C	56	30	No hits				
33		18718	18885		55	40	No hits				
34		2514	2681	C	55	18	No hits				
35		10104	10265	C	53	41	No hits				
36		11177	11335		52	30	No hits				
37	*efae1204*	10643	10801		52	35	Hypothetical protein EfaeDRAFT 1204 (*E. faecium*)	29/48	EAN09492	3-47	27-73 (207)
38		10441	10596	C	51	35	No hits				
39		2709	2864		51	24	No hits				
40		4304	4450	C	48	23	No hits				
41		19866	20006	C	46	31	No hits				
42		9446	9586		46	31	No hits				
43		16832	16966		44	36	No hits				
44		288	422	C	44	40	No hits				
45	*entI(L50A)*	2363	2497	C	44	30	Enterocin I (Enterocin L50A) (*E. faecium*)		CAA76199		
46	*entJ(L50B)*	2212	2343	C	43	33	Enterocin J (Enterocin L50B) (*E. faecium*)		CAA76200		

aas = amino acids.

[a]C, complementary sequence.